

Glucose Regulates the Release of Orexin-A From the Endocrine Pancreas

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Orexins (hypocretins) are novel neuropeptides that appear to play a role in the regulation of energy balances. Orexin-A (OXA) increases food intake in rodents, and fasting activates OXA neurons in both the lateral hypothalamic area and gut. OXA is also found in the endocrine pancreas; however, little is known about its release or functional significance. In this study, we show that depolarizing stimuli evoke the release of OXA from rat pancreatic islets in a calcium-dependent manner. Moreover, OXA release is stimulated by low glucose (2.8 mmol/l), similar to glucagon secretion, and inhibited by high glucose (16.7 mmol/l). Fasting increases plasma OXA, supporting the idea that orexin is released in response to hypoglycemia. Cells that secrete glucagon and insulin contain OXA and both cell types express orexin receptors. OXA increases glucagon secretion and decreases glucose-stimulated insulin release from isolated islets. OXA infusion increases plasma glucagon and glucose levels and decreases plasma insulin in fasted rats. We conclude that orexin-containing islet cells, like those in the brain and gut, are glucosensitive and part of a network of glucose "sensing" cells that becomes activated when blood glucose levels fall. OXA may modulate islet hormone secretion to maintain blood glucose levels during fasting. *Diabetes* 52: 111–117, 2003

Orexins (hypocretins) are novel neuropeptides that play a role in arousal and the regulation of energy balances (1–3). Orexin-A (OXA) and -B are 33- and 28-residue peptides, respectively, that are isolated from the rat hypothalamus and named for their ability to stimulate feeding when injected into the brain (2). Their common precursor, prepro-orexin, is expressed in a specific population of neurons in and around the lateral hypothalamic area (LHA) (2), a region classically implicated in the control of mammalian feeding behavior (4). The LHA contains glucosensitive neurons that are stimulated by falls in circulating glucose (5) and

inhibited by prandial signals, such as the presence of food in the gut and/or a rise in portal glucose concentration (4). Previous studies have suggested that orexin neurons might correspond to this neuronal population, since orexin neurons are activated by hypoglycemia (6) and increased hypothalamic prepro-orexin mRNA levels are observed in response to fasting (1,7); however, glucosensitive neurons do not contain OXA (8). They are excited by the peptide (8), implying that orexin acts as a neuromodulator at glucosensitive cells, stimulating them under conditions of hunger.

Glucosensitive neurons are also found in regions outside the hypothalamus, including the nucleus of the solitary tract (NTS) (9) and enteric nervous system (ENS) (10). The NTS relays visceral afferent signals to the LHA (8). Orexin fibers are distributed extensively in the NTS (11), and glucosensitive neurons are activated by the same conditions that stimulate orexin neurons in the LHA (6). The ENS participates in the regulation of food intake by directly sensing, integrating, and regulating the machinery of the gut involved in energy metabolism (3). We have previously shown that orexin neurons are located throughout the ENS and, like their hypothalamic counterparts, are activated by fasting (12). Orexins may sense nutritional status in the gut, modulating secretion and motility in order to prime the digestive tract in preparation for food ingestion and energy uptake. Endocrine cells in the gastric and intestinal mucosa (12,13), as well as in the pancreas (12), also contain orexins; therefore, orexins may function as circulating hormones and/or paracrine or autocrine transmitters. The presence of OXA in human plasma supports this idea (14,15).

Little is known about the regulation and role of the orexins in the endocrine pancreas. We have previously shown that OXA and orexin receptor immunoreactivity is displayed by the insulin-secreting β -cells in the rat pancreas (12). OXA release in the extracellular medium might allow β -cells to regulate neighboring cells that express orexin receptors. The present study was conducted to determine whether 1) endogenous OXA is secreted from rat pancreatic islets, 2) OXA release is modulated by extracellular glucose concentrations, and 3) OXA affects pancreatic hormone secretion.

RESEARCH DESIGN AND METHODS

Animals. Adult female or male Sprague-Dawley rats (150–300 g) were used. Animals were killed by CO₂ inhalation and exsanguinated. The Animal Care and Use Committee of the State University of New York Downstate Medical Center and the local ethics committee for animal experimentation in Stockholm, Sweden, approved the experimental protocols.

Isolation and culture of pancreatic islets. Pancreatic islets were isolated by digestion with collagenase as previously described (16). Islets were

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Received for publication 19 June 2002 and accepted in revised form 9 October 2002.

A.L.K. is employed by and holds stock in GlaxoSmithKline. EIA, enzyme immunoassay; ENS, enteric nervous system; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; IR-OXA, immunoreactive Orexin-A; LHA, lateral hypothalamic area; NTS, nucleus of the solitary tract; OXA, Orexin-A; OXR1, orexin receptor 1; TFA, trifluoroacetic acid; TTX, tetrodotoxin.

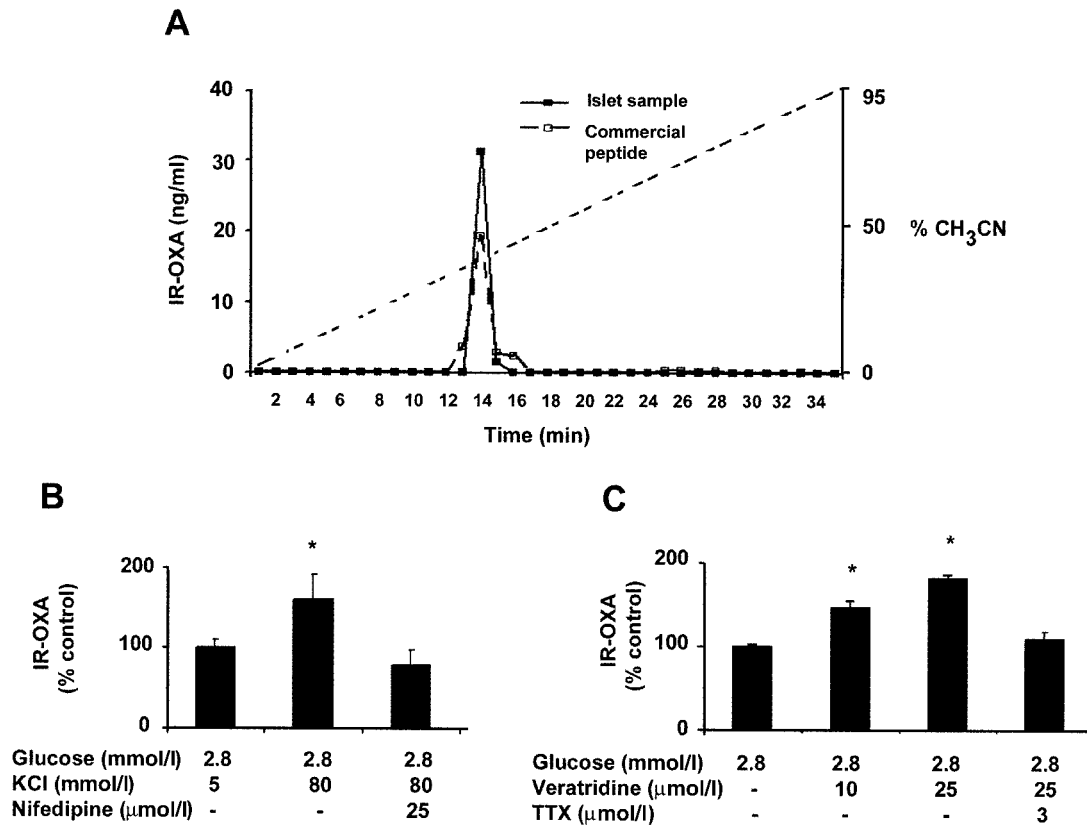


FIG. 1. Effects of depolarizing stimuli on OXA release from islets isolated from the rat pancreas. **A:** Reversed-phase HPLC. Islet IR-OXA elutes as a single peak that is superimposable with the peak observed with commercial rat OXA. **B:** High KCl (80 mmol/l) increases the release of IR-OXA. The effect of high KCl is completely blocked by the L-type Ca²⁺ channel blocker nifedipine (25 μmol/l). **C:** Veratridine increases the release of IR-OXA in a concentration-dependent manner, and the effect is blocked by TTX (3 μmol/l). Results are expressed as percentages of control IR-OXA release in the presence of 2.8 mmol/l glucose (2.22 ± 0.42 fmol · islet⁻¹ · min⁻¹, *n* = 8). **P* < 0.05 vs. control.

selected under a dissecting microscope, transferred to Petri dishes, and cultured for 20–22 h in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10 mmol/l glucose.

Static incubation of pancreatic islets. Incubation of pancreatic islets was performed as previously described (16,17). Briefly, groups of 10 islets were preincubated in 1 ml of Krebs bicarbonate-buffered solution (115 mmol/l NaCl, 5 mmol/l KCl, 2.56 mmol/l CaCl₂, 1 mmol/l MgCl₂, and 24 mmol/l NaHCO₃) containing 0.5% (wt/vol) albumin (fraction V, radioimmunoassay grade; Sigma Chemical, St. Louis, MO) and 2.8 mmol/l glucose for 30 min at 37°C. The preincubation medium was aspirated and replaced with the same medium in the absence and presence of test compounds. Final incubations were carried out for 90 min at 37°C in a shaking water bath. At the end of the test incubations, the medium was aspirated and stored at -20°C. The effects of high KCl, veratridine (Sigma Chemical), OXA (Peninsula Laboratories, San Carlos, CA), and glucose were studied. In experiments with tetrodotoxin (TTX), diazoxide, and nifedipine (Sigma Chemical), the test substances were present in the medium during both the prestimulation and stimulation incubations.

OXA extraction and measurement. Samples were loaded onto a Sep-Pak C18 cartridge (Peninsula Laboratories), which was pretreated with acetonitrile (CH₃CN) and then washed with 0.1% trifluoroacetic acid (TFA). After washing with TFA, adsorbed peptides in the cartridge were eluted with 60% CH₃CN/0.1% TFA. The eluate was dried under vacuum in a Speed Vac SC100 Savant apparatus and stored at -20°C until the time of OXA assay.

OXA was measured in triplicate using a commercial enzyme immunoassay (EIA) kit (Peninsula Labs) that uses a rabbit polyclonal antibody raised against human OXA. The antibody does not cross-react with orexin-B, neuropeptide Y, or leptin. OXA concentrations were determined against a known standard (0–25 ng/ml), and the detection limit of the assay was 60–80 pg/ml of sample.

Reversed-phase high-performance liquid chromatography. Chromatographic characterization of the extracts was performed by reversed-phase high-performance liquid chromatography (HPLC) using an Aquapore C4 column (BU-300, 7μ 30 × 4.6 mm; Aquapore, Bodman, PA). The column was eluted with a linear gradient of CH₃CN containing 0.1% TFA at a flow rate of

0.7 ml/min. Fractions (0.7 ml) were collected continuously. Each fraction was dried, reconstituted with assay buffer, and assayed.

OXA infusion. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.; Apoteksbolaget, Stockholm, Sweden) and implanted with a jugular vein catheter for administration of drugs. The catheter was tunneled subcutaneously to exit at the back of the animal's neck. The rats were housed singly and allowed to recover for at least 7 days before experiments were undertaken. During recovery, the rats were trained to accept experimental conditions. Experiments were then carried out in conscious animals placed in Bollman cages after an 18-h fasting period with free access to water. OXA (100 pmol · kg⁻¹ · min⁻¹; Bachem AG, Bubendorf, Switzerland) or vehicle was administered for 20 min using a microinjection pump (CMA 100; CMA Microdialysis, Sölna, Sweden). A blood sample was obtained at the termination of the infusion, and insulin and glucagon were measured as described below.

For glycemia studies, OXA (80 pmol · kg⁻¹ · min⁻¹) was infused for 60 min. Blood samples were taken at 20-min intervals before, during, and after OXA injection. Vehicle was administered as a control.

Blood sampling. Blood was collected in propylene tubes containing EDTA (1 mg/ml blood) and aprotinin (500 KIU/ml), centrifuged, and then stored at -80°C. Plasma-immunoreactive OXA, insulin, and glucagon levels were measured in triplicate. Blood glucose was determined by the glucose oxidase method using a glucose analyzer.

Glucagon and insulin measurement. Glucagon was measured using a rat glucagon EIA kit (Peninsula Labs) and human glucagon as standard. Glucagon concentrations were determined against a known standard (0–25 ng/ml), and the detection limit of the assay was 60–80 pg/ml of sample. Insulin was quantified using High Range Rat Insulin ELISA kit (ALPCO Diagnostics), with rat insulin as standard. The range for insulin measurement was 0–125 ng/ml with a limit of ~3 ng/ml.

Immunocytochemistry. The pancreas was fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4), embedded in OCT (TissueTek), and sectioned (10 μm) using a cryostat-microtome. To locate proteins in the tissue by immunocytochemistry, preparations were exposed to PBS containing 1% Triton X-100 and 4% horse serum for 30 min. Primary antibodies were against OXA (affinity-purified rabbit polyclonal, diluted 1:500; Alpha Diagnostic Inter-

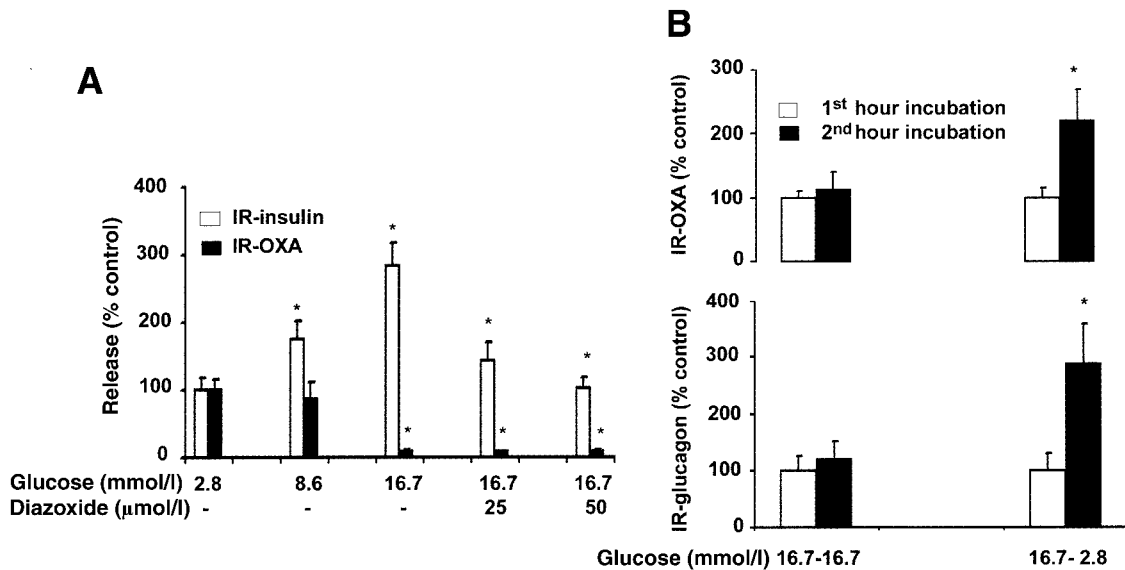


FIG. 2. Effects of glucose on IR-OXA, immunoreactive glucagon, and immunoreactive insulin release from isolated pancreatic islets. **A:** High glucose (16.7 mmol/l) inhibits the release of OXA. In contrast, glucose stimulates the release of insulin and the effect is dose dependently reduced by the ATP-sensitive K^+ channel opener diazoxide. Diazoxide has no effect on the release of OXA. **B:** Low glucose (2.8 mmol/l) stimulates the release of both OXA and glucagon. Results are expressed as percentages of control IR-OXA (2.22 ± 0.42 fmol \cdot islet $^{-1} \cdot$ min $^{-1}$, $n = 8$), immunoreactive (IR) glucagon (0.38 ± 0.10 fmol \cdot islet $^{-1} \cdot$ min $^{-1}$, $n = 8$), or immunoreactive insulin (2.10 ± 0.33 fmol \cdot islet $^{-1} \cdot$ min $^{-1}$, $n = 8$) release in the presence of 2.8 mmol/l. * $P < 0.05$ vs. control.

national, San Antonio, TX) and orexin receptor 1 (OXR1) (affinity-purified rabbit polyclonal, diluted 1:500; Alpha Diagnostic International) (13) followed by incubation with donkey anti-rabbit secondary antibodies coupled with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:500 for 3 h. Double labeling was made possible by using primary antibodies raised in different species in conjunction with species-specific secondary antibodies (donkey anti-mouse, donkey anti-guinea pig; Jackson ImmunoResearch Labs) coupled to contrasting fluorophores FITC or Cy 3 (Kirkegaard and Perry, Gaithersburg, MD). Primary antibodies were against insulin (mouse monoclonal, diluted 1:1,000; ICN ImmunoBiologicals, Lisle, IL) and glucagon (guinea pig polyclonal, diluted 1:1,000; Linco Research). The sections were coverslipped with Vectashield (Vector Labs, Burlingame, CA). No staining was observed in control sections incubated without primary antibody. Sections were examined by using a Radiance 2000 laser scanning confocal microscope (Bio-Rad, San Francisco, CA) attached to an Axioskop 2 microscope (Zeiss, New York).

Data analysis. Data are expressed as means \pm SE. Statistical significance was evaluated using ANOVA followed by ad hoc analysis using Tukey's test. Differences were considered significant at $P < 0.05$.

RESULTS

Depolarizing stimuli evoke the release of OXA. In the first part of this study, we characterized the in vitro release of immunoreactive OXA (IR-OXA) from isolated islets in response to various depolarizing stimuli. Basal IR-OXA release in the presence of 2.8 mmol/l glucose was 2.67 ± 0.28 fmole \cdot islet $^{-1} \cdot$ min $^{-1}$ ($n = 8$). When characterized by reversed-phase HPLC, islet IR-OXA eluted as a peak that was superimposable with the peak of synthetic OXA (Fig. 1A). Depolarization of the incubated islets with 80 mmol/l KCl induced a 61% ($n = 8$, $P < 0.05$) increase in IR-OXA release over the basal level (Fig. 1B), suggesting that at least some OXA is stored and released via a regulated pathway. The release of IR-OXA was also significantly increased by the alkaloid veratridine, a Na^+ channel activator (Fig. 1C). Veratridine (10 μ mol/l) induced a 47% ($n = 5$, $P < 0.05$) increase in the release of IR-OXA over the mean basal level. The effect of veratridine was concentration dependent and blocked by the Na^+ channel inhibitor TTX (3 μ mol/l; $109.43 \pm 8.29\%$ vs. $100 \pm 2\%$ of

basal level, $n = 6$) (Fig. 1C). IR-OXA release produced by high KCl (80 mmol/l) was completely inhibited by pretreating islets with the L-type Ca^{2+} channel blocker nifedipine (25 μ mol/l, $n = 8$) (Fig. 1B). Thus, the stimulated release of OXA depends on membrane depolarization and Ca^{2+} influx through L-type Ca^{2+} channels.

The release of OXA is regulated by glucose. Next, we examined whether the release of OXA from islets is regulated by extracellular glucose concentrations. An increase of the glucose concentration from 2.8 to 16.7 mmol/l caused a significant decrease in islet IR-OXA release from 100.00 ± 15.67 to $8.20 \pm 2.07\%$ of control ($P < 0.05$) (Fig. 2A). Insulin release was determined from the same samples that were used to determine OXA release. Insulin secretion increased from 100.00 ± 18.98 to $283.58 \pm 35.34\%$ of control ($n = 8$, $P < 0.05$) (Fig. 2A). The finding that IR-OXA release was still reduced in the presence of the ATP-sensitive K^+ channel opener diazoxide (25 and 50 μ mol/l) (Fig. 2A) indicates that the inhibitory effect of glucose is not due to insulin- or glucose-induced membrane depolarization.

Conversely, when the glucose concentration was decreased from 16.7 to 2.8 mmol/l, IR-OXA release significantly increased from 100.00 ± 15.95 to $218.96 \pm 49.86\%$ ($P < 0.05$) (Fig. 2B, top). As expected, glucagon release was also significantly increased (from 100.00 ± 30.17 to 288.27% , $P < 0.05$) (Fig. 2B, bottom). Thus, a negative relationship between glucose concentration and OXA release by pancreatic islets was observed in the present study.

Plasma OXA is increased during fasting. Since the release of OXA is stimulated by low glucose, we hypothesized that the release of OXA is increased during fasting. To test this idea, IR-OXA was measured in the plasma of fasted rats. Plasma IR-OXA eluted as a single peak that was superimposable with the peak of islet release (data not

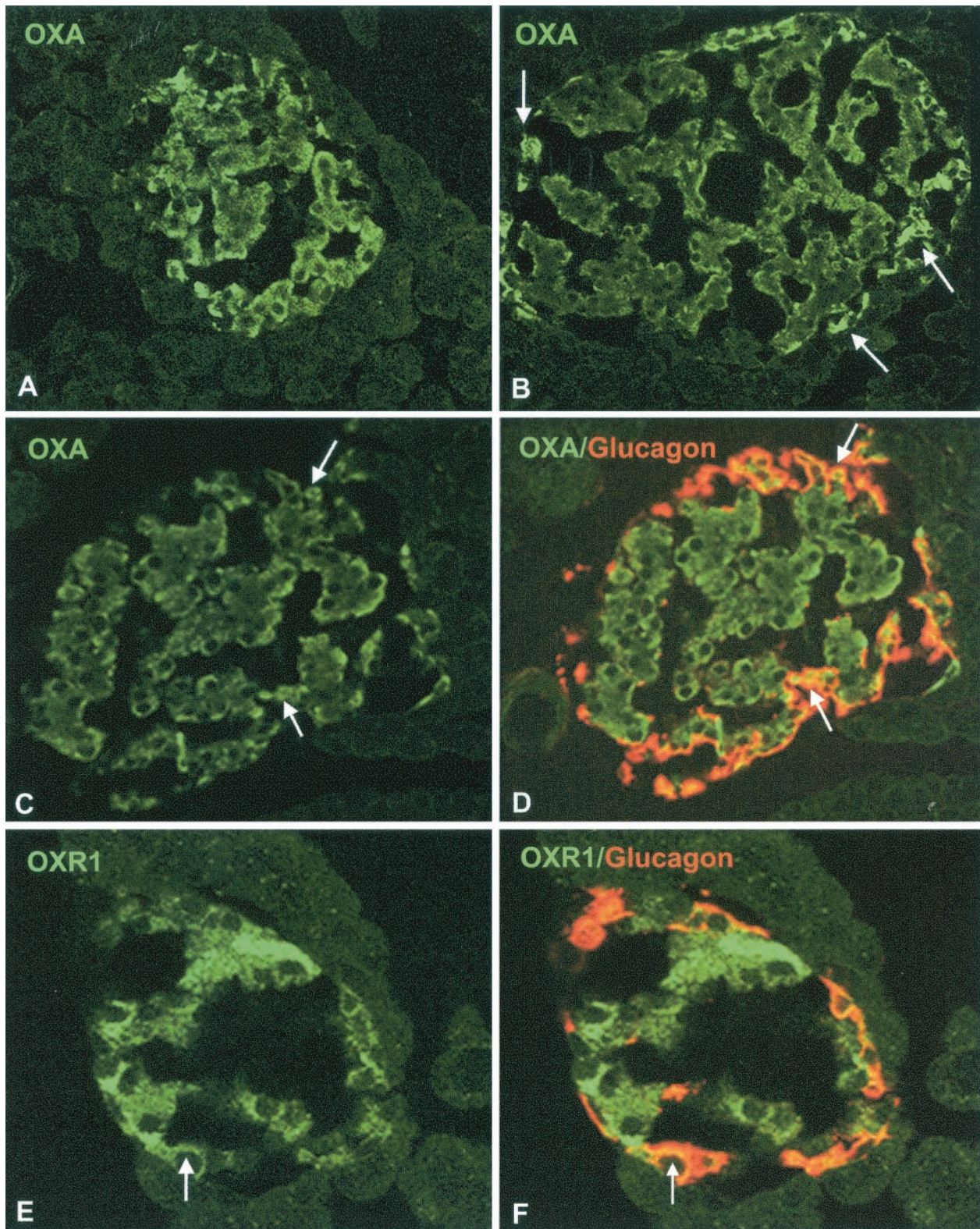


FIG. 3. Localization of OXA and OXR1 in pancreatic islets. *A–F*: Cryostat sections of rat pancreas were immunostained for OXA (green) and glucagon (red). Specific staining is observed in a subset of cells (arrow). The merged confocal images (*D* and *F*) demonstrate the presence of OXA and OXR1 in glucagon-secreting islet cells (yellow, arrow). Note that non-glucagon-containing cells also display peptide and receptor immunoreactivity. Scale bars, 15 μ m.

shown). Food deprivation resulted in a significant ($P < 0.05$) rise in plasma IR-OXA (from 33.1 ± 6.4 to 53.4 ± 11.5 pg/ml, $n = 6$). These findings are consistent with the hypothesis of increased OXA release during fasting.

Islet cells display OXA and orexin receptor immunoreactivity. The increase in release of islet IR-OXA and glucagon by low glucose prompted us to determine whether the same cells produce OXA and glucagon. As

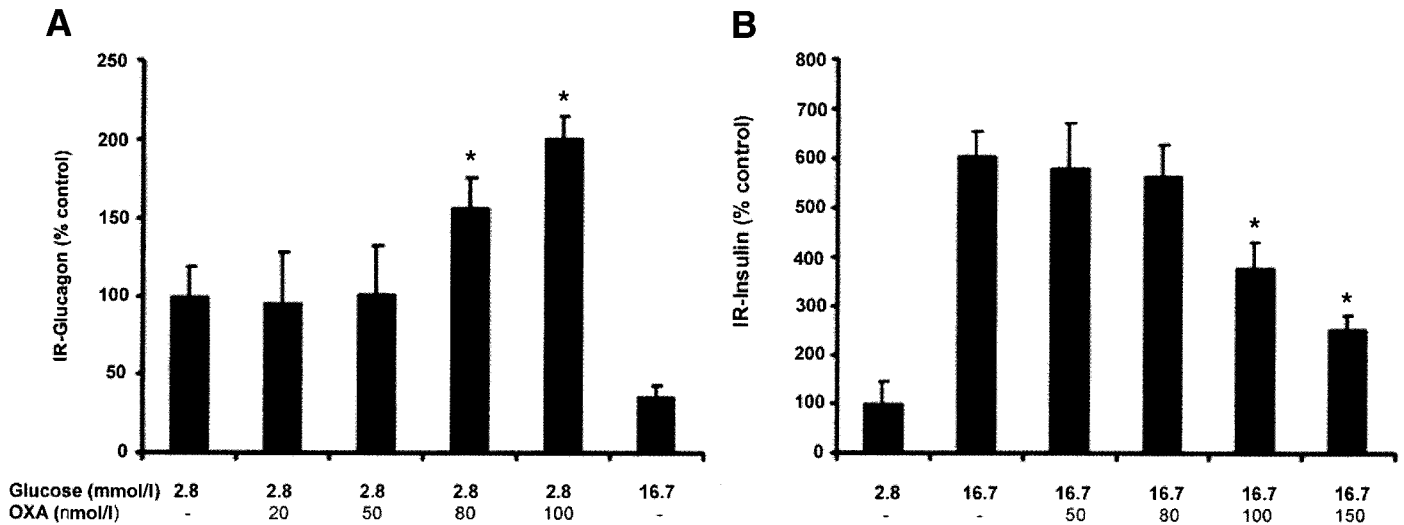


FIG. 4. Effect of OXA on glucagon and insulin secretion from isolated pancreatic islets. *A*: OXA (80 and 100 nmol/l) potentiates the release of glucagon from rat islets in the presence of low glucose (2.8 mmol/l). *B*: OXA (100 and 150 nmol/l) inhibits glucose (16.7 mmol/l)-stimulated insulin secretion. Results are expressed as percentages of control immunoreactive (IR) glucagon ($0.21 \pm 0.04 \text{ fmol} \cdot \text{islet}^{-1} \cdot \text{min}^{-1}$, $n = 8$) or insulin ($1.96 \pm 0.88 \text{ fmol} \cdot \text{islet}^{-1} \cdot \text{min}^{-1}$, $n = 8$) release in the presence of 2.8 mmol/l glucose. * $P < 0.05$ vs. without drug.

previously reported (12), OXA immunoreactivity was observed in the cytoplasm of β -cells (Fig. 3A); however, intense OXA immunoreactivity was also observed in cells located at the periphery of a subset of islets (Fig. 3B). Double labeling with antibodies to OXA and glucagon revealed that OXA immunoreactivity is displayed by the glucagon-secreting α -cells (Figs. 3C and D). In addition, both β - and α -cells display OXR1 immunoreactivity (12) (Figs. 3E and F), suggesting that OXA might affect insulin and/or glucagon secretion.

OXA modulates glucagon and insulin secretion from isolated islets. We examined the effects of OXA on hormone release from isolated islets. OXA (100 nmol/l) significantly ($P < 0.001$) increased glucagon secretion (from 100 ± 17 to $200.74 \pm 13.47\%$, $n = 8$) from islets incubated in low (2.8 mmol/l) glucose (Fig. 4A) and had no effect on the insulin level (not shown). A significant increase ($P < 0.05$) in glucagon secretion was also observed in the presence of 80 nmol/l OXA; however, no

effect was observed at concentrations in the range of 1–50 nmol/l (Fig. 4A).

When the glucose concentration was raised to 16.7 mmol/l, OXA (100 nmol/l) significantly ($P < 0.05$) decreased glucose-stimulated insulin secretion (605.93 ± 48.26 to $375.38 \pm 52.95\%$, $n = 8$) (Fig. 4B). A significant ($P < 0.001$) decrease in glucose-stimulated insulin secretion was also observed in the presence of 150 nmol/l of OXA (from 252.48 ± 25.66 to $605.93 \pm 48.26\%$) (Fig. 4B); however, no effect was observed at concentrations in the range of 1–80 nmol/l (Fig. 4B).

OXA modulates plasma insulin, glucagon, and glucose in fasted rats. Next, we determined whether OXA modulates plasma insulin, glucagon, and/or glucose level. Like the exogenous application of OXA to islets incubated in low glucose, the intravenous administration of the peptide ($100 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 20 min) significantly ($P < 0.05$) increased plasma glucagon in fasted rats (from 33.25 ± 2.14 to $53.5 \pm 6.5 \text{ pg/ml}$, $n = 4$). In contrast,

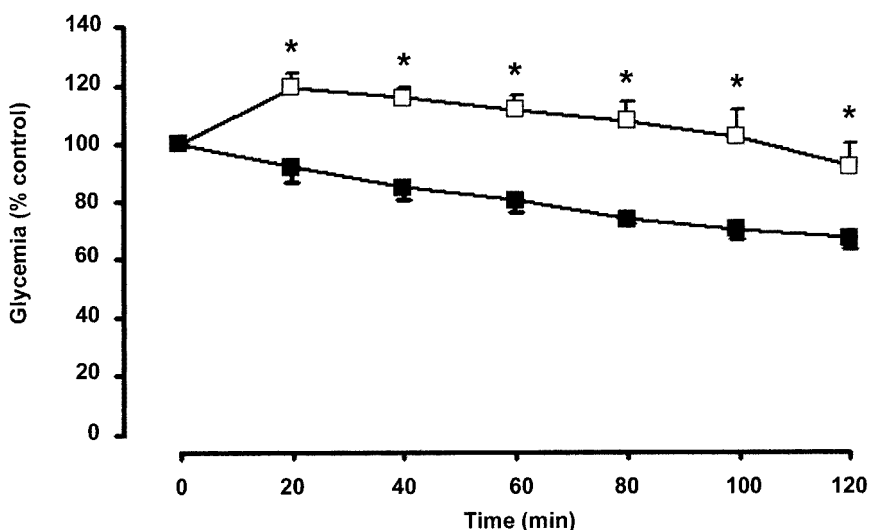


FIG. 5. Effect of OXA on blood glucose levels. Animals were food deprived for 12 h before infusion of vehicle (isotonic saline; ■) or OXA ($80 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; □). Glucose concentrations were measured every 20 min. Time 0 corresponds to the beginning of vehicle or OXA infusion. Blood glucose levels are expressed as percentages of mean blood glucose concentration observed at time 0. Values represent means \pm SE of five independent experiments. * $P < 0.05$ vs. control.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health Grant NS27645, the American Diabetes Association, and the Swedish Research council.

We thank Jimmy Calaycay and Dr. Rushbrook for their guidance with HPLC and comments on the manuscript and Dr. Gintzler for his advice on protein extraction. We also thank Dr. Bianchi for comments on the manuscript and Jie Ma and Qingchun Tong for assistance with immunocytochemistry and animal surgery.

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